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14. ABSTRACT Hyaluronan is a high molecular weight polyanionic polysaccharide that is increased in more advanced prostate cancers. Tumor cell interaction with this polysaccharide by specific receptors CD44 and RHAMM promote tumor growth, survival and invasion. Work during the last funding period have further defined the mechanism of action of each of these receptors. Studies show that extracellular RHAMM acts a co-receptor for CD44, and the combined action of this receptor complex leads to sustained activation of the ERK 1,2 signal transduction pathway leading to enhance motility and produce patterns of gene transcription that are associated with invasion. Synthetic peptides have also been identified that can bind hyaluronan and inhibit the binding of this polysaccharide to its cognate receptors. These peptides inhibit tumor growth both in vitro and in vivo and the residues important for the activity of the peptides are being defined using nuclear magnetic resonance (NMR). Small molecule libraries that contain compounds which may mimic these peptides are also being interrogated for the ability to inhibit hyaluronan binding to RHAMM and CD44 and to inhibit tumor growth. The goal is to develop new therapeutic strategies for patients with invasive prostate cancer.

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Table of Contents

Introduction:

This 3rd annual report contains much of what was included in the previous annual report to faciliatate a clearer understanding of the progress made during the first 3 years of funding. The last year of funding has been largely dedicated to generating specific antibodies and synthetic peptides that will be used during year 4 (no cost extension). The activities during year 3 are highlighted in red text.

The focus of this grant is to evaluate the importance of hyaluronan and tumor cell receptors for hyaluronan in promoting tumor progression. Hyaluronan is a high molecular weight (10^5-10^7 daltons) anionic carbohydrate that is associated with the progression of a number of cancers, including prostate cancer (Simpson and Lokeshwar, 2008; Toole, 2004). Tumor associated hyaluronan first appears in the tumor reactive stroma, whereas later stages of progression are also associated with an increase in synthesis by the epithelial compartment of the tumor (Simpson and Lokeshwar, 2008). Furthermore, tumor associated hyaluronan is also fragmented, in part by an upregulation of hyaluronidases, which create smaller oligomers that have different biological properties than the high molecular weight polymer. One important difference in the biological properties of lower molecular weight oligomers (~3 X 10^3 daltons) is that such oligomers will stimulate angiogenesis, whereas higher molecular polymers impede angiogenesis. Furthermore, hyaluronan/tumor cell interactions enhance tumor cell survival, drug resistance and increase both growth and invasion by mechanisms that are not yet completely understood (Toole, 2004; Toole et al., 2005). Importantly, strategies to disrupt tumor cell/hyaluronan interactions do offer the potential to impede tumor progression and enhance sensitivity to current therapies. This is the underlying rationale for pursuing the studies funded by this grant award.

The two hyaluronan receptors expressed by invasive/metastatic prostate tumor cells are CD44 and RHAMM (CD168), and the funded studies focused on understanding the relative importance of each receptor in the biology of prostate tumor progression. There have been substantial changes to the understanding of RHAMM function since this initial award was made. We summarized some of these results in a recent opinion paper published by the PI and co-PI in Journal of Cell Science.((Maxwell et al., 2008)). This JCS paper documents the history of studies of RHAMM function, which include its original discovery as an extracellular motogen receptor for hyaluronan (Turley et al., 2002). The paper also highlights other studies demonstrating that RHAMM has intracellular functions, which include binding to interphase microtubules, localizing within the nucleus, and participating in mitotic spindle formation (Assmann et al., 1999; Maxwell et al., 2003; Turley et al., 2002). Dysregulation of intracellular RHAMM levels in cells has also been proposed to contribute to genomic instability in cells (Joukov et al., 2006; Pujana et al., 2007). We have also recently finalized a chapter for Seminars in Cancer Biology that summarizes the entirety of our findings that were in large part funded by the DOD award. This chapter is included in the Appendix and does credit the current award (McCarthy and Turley, Appendix 1). Two additional peer reviewed papers, which have been funded by the DOD award, have also also been published during the funding period (Tolg, et al. 2006; Hamilton, et al. 2007). In these studies, models in

addition to prostate cancer have been characterized for RHAMM function since each model has its advantage compared to the prostate cancer cell lines being used in the funded award. These studies have a direct bearing on changes in the original design of studies to use prostate cancer cell lines in these studies. One of these papers utilizes a RHAMM -/- animal model to specifically address the function of extracellular RHAMM, since the focus of the current prostate cancer proposal is to address the hyaluronan related properties of this motogen. The second paper utilized breast cancer model cell lines that differ in the expression of cell surface RHAMM and cell surface hyaluronan. The conclusions from these studies have been essential for creating a testable working model for RHAMM function in prostate.

The funded proposal has three specific aims which are:

Specific Aim 1: To evaluate the ability of hyaluronan to enhance tumor growth and invasion in the presence or absence of CD44 and/or RHAMM.

Specific Aim 2: ***To identify structural features of CD44 and RHAMM that mediate the effects of hyaluronan on signaling pathways regulating tumor growth, survival and invasion.***

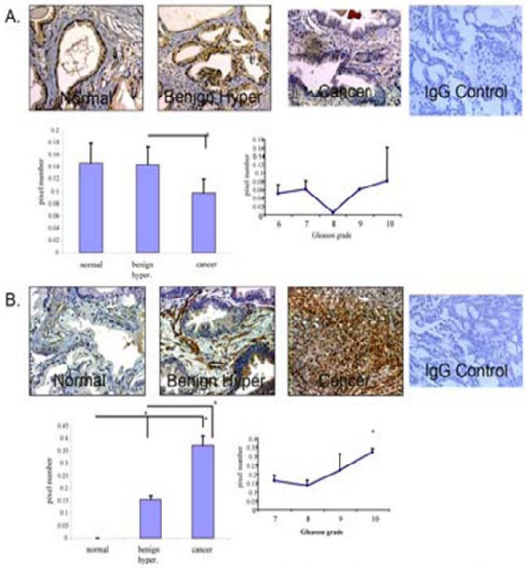
Specific Aim 3: ***To test the ability of specific hyaluronan-binding synthetic peptides to inhibit hyaluronan binding to CD44 and RHAMM or tumor growth/survival in vitro and in vivo.***

The next section addresses the state of progress on these studies as organized by the original Statement of Work.

Body

Statement of Work:
Aim 1: ***to evaluate the ability of hyaluronan to enhance tumor growth and invasion in the presence or absence of CD44 and/or RHAMM***

Task 1: Determine the effect of inhibiting CD44 or RHAMM function/expression on stimulating the growth, invasion and motility of PC3M-LN4 cells.

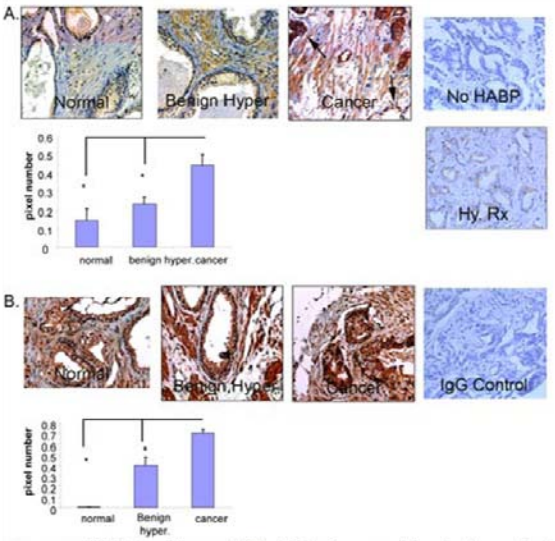


. CD44 (A) and RHAMM (B) expression in human tissue arrays. Staining intensity was determined by densitometry with values representing means ± SEM from 2 separate arrays. Measurements in triplicate were compared using a students t test and asterisks mark p<0.01.

Although a study of human tissues was not originally part of the work statement, comments were made during the review that relevance to human disease had not been clearly established, and so we have first embarked on a more systematic study to evaluate the relationship between CD44, RHAMM, hyaluronan (HA) and hyaluronidase 1 expression (Hyase 1).

Human tissue microarrays were used to first evaluate the levels of CD44 (Figure 2A), RHAMM (gene name, HMMR; Figure 2B), HA (Figure 3A) or Hyal-1 (Figure 3B) in human tumors. The tissue microarrays each had 40 samples equally divided between benign hyperplasia (BPH) and different Gleason Grades (6-10), along with two normal controls. Anti-RHAMM antibodies were generated against a synthetic peptide from residues 169 to 182 (ELMKLRNKRETKMR) and the IgG purified from immune or prebleed sera as described (Appendix 2 and 3) and specificity was confirmed by Western blot of PC3M-LN4 cell extracts vs. RHAMM -/- fibroblast extracts or recombinant RHAMM protein (not shown). The tissue sections on the microarray were scored for Gleason Grade and initially the staining intensity of both tumor stroma and epithelium were ranked by Dr. Steve Schmechel (surgical pathologist, U of MN), then intensity in the epithelial compartment was quantified using image analysis and densitometry.

The level of tumor associated CD44 staining overall was reduced in cancer samples compared to normal tissue or BPH and it remained lower but did not vary as a function of Gleason score (Figure 2A). In contrast, RHAMM was not detected in normal tissue but was elevated in BPH, and most strongly elevated in prostate carcinomas (Figure 2B). The intensity of RHAMM staining increased as a function of Gleason Score (Figure 2B). Elevated levels of both HA (Figure 3A) and Hyal-1(Figure 3B) were also observed in both BPH and prostate cancer, with a statistically higher staining intensity detected in the carcinoma compared to normal or BPH epithelia. The staining intensity of both HA and Hyase-1 also increased as a function of Gleason Scores (not shown). Dr. George Vasmatzis (Bioinformatics, Mayo Clinic) has also provided data obtained using microarray expression analysis of laser capture microdissected tumors comparing normal and tumor cells within individual tumors (personal communication). His initial findings from the microarray data indicate that RHAMM expression in the carcinoma increases is associated with aggressiveness (p-value 0.04) and is independent of Gleason



HA (A) and hyase 1 (B) staining in normal, benign hyperplasia and prostate cancer. Both HA and hyase 1 accumulation/expression increase significantly (P<0.01) above normal and benign hyperplasia levels in prostate Cancer. There is a trend for increased HA accumulation with higher Gleason scores.

Score. Furthermore, RHAMM expression is very high in 6/7 metastases (nodal metastasis cases), implicating the expression of this gene in malignant progression. Collectively, these data, and the studies summarized below (included in the Appendix), strongly support the rationale for evaluating the cooperation of RHAMM and CD44 in a hyaluronan/hyaluronidase rich environment in primary or metastatic human prostate tumors.

Studies have been initiated to evaluate the relative importance of RHAMM and CD44 in promoting prostate tumor cell migration. Some of these studies have utilized a RHAMM -/- mouse, generated by Dr. Turley ((Tolg et al., 2006), Appendix 2). The reason for utilizing this mouse model, developed by Dr. Turley, is related in part to the realization that RHAMM, which functions as an extracellular hyaluronan receptor, also has intracellular functions which include binding to microtubules, participating in mitotic spindle assembly and it can also localize to the nucleus (Assmann et al., 1999; Maxwell et al., 2003; Maxwell et al., 2008; Turley et al., 2002). Indeed, recent studies by Livingston's group have implicated dysregulation of RHAMM levels (due to defects in BRCA1 E3 ubiquitin ligase activity) in abnormal spindle formation (Joukov et al., 2006; Pujana et al., 2007).

The conclusions of the published knockout animal studies are summarized as follows:

1. RHAMM -/- animals exhibit defects in excisional wound healing.

2. The inflammatory response is abnormal in wound of RHAMM -/- animals
    a. The granulation tissue of RHAMM -/- wounds is abnormal compared to wild type controls
    b. Neutrophils persist longer than in wild type animals
    c. There is a defect in monocyte/macrophage infiltration into the granulation tissue

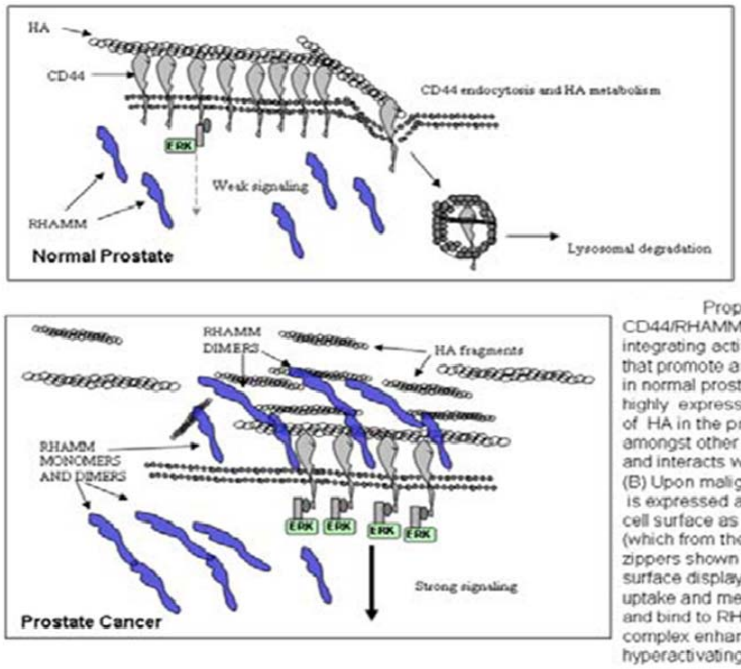
3. Fibrogenesis/repair in RHAMM -/- animals is also defective
    a. Fibroblasts from RHAMM -/- have defective migratory properties both in vivo and in vitro
    b. The sustained activation of ERK 1,2, which has been associated with extracellular RHAMM function, does not occur in RHAMM -/- fibroblasts in vivo or in vitro
    c. The defective phenotype can be rescued with beads to which recombinant RHAMM has been attached. This was used to avoid complications of internalization of RHAMM in the RHAMM -/- cells. The rescued motility phenotype was sensitive to inhibition by MEK1 inhibitors
    d. Recombinant RHAMM coupled beads also rescued sustained activation of the ERK 1,2 pathway leading to nuclear localization of pERK 1,2

4. Extracellular RHAMM mediates motility and sustained activation of the ERK 1,2 pathway by enhancing cell surface localization of CD44
    a. CD44 colocalizes with RHAMM coated beads on the surface of RHAMM -/- fibroblasts
    b. Antibodies against CD44 inhibit wild type fibroblasts inhibits hyaluronan stimulated motility. These antibodies also inhibit the enhance migration of RHAMM -/- fibroblasts stimulated with RHAMM coated beads
    c. RHAMM -/- CD-/- cells do not enhance migration in response to HA and are not rescued by RHAMM coated beads

Therefore, we conclude from this study that extracellular RHAMM can stimulate motility in the absence of intracellular RHAMM, and that extracellular RHAMM works in conjunction with CD44 to stimulate motility and enhance the intensity and duration of activation of the ERK 1, 2 pathway. We hypothesize that this RHAMM-enhanced activation of ERK 1, 2 via CD44 is at least partly due to enhanced cell surface retention of CD44.

A second publication credited to the DOD award published in J. BIol. Chem. (Hamilton et al., 2007) utilized well characterized breast tumor cell lines for motility and RHAMM expression. Two paired cell lines with differing invasive properties were compared for RHAMM, CD44 and HA expression. The studies have led to our constructing a model for RHAMM, CD44, hyaluronan and ERK 1, 2 activation in prostate cancer cells. The findings in the J. Biol. Chem. paper are as follows:

1. Highly motile/invasive breast cancer cell lines MDA-MB-231 or Ras transfected MCF 10A cells express cell surface RHAMM, CD44, hyaluronan and exhibit sustained activation of the ERK 1,2 pathway compared to poorly invasive/migratory MCF7 or parental MCF 10A cells.
2. RHAMM, CD44 and active ERK 1, 2 co-precipitate from the highly migratory cell lines indicative of a macromolecular signaling complex.
3. Combinations of antibodies against CD44, RHAMM or a MEK1 inhibitor were less than additive at blocking motility, suggesting all three are involved in a common motogenic pathway
4. Although not specifically tested in this system, the presence of extracellular hyaluronan in the highly migratory cells and the common hyaluronan binding properties of both CD44 and RHAMM have led us to propose that CD44 can pair, via hyaluronan, to cell surface RHAMM to form a signaling complex. A schematic of that model is shown below:



Proposed model for roles of CD44/RHAMM/HA interactions for integrating activation of signaling cascades that promote and sustain prostate tumorigenesis. in normal prostate tissue (A) CD44 protein is highly expressed and acts to maintain low levels of HA in the prostate cell microenvironment amongst other functions. RHAMM is intracellular and interacts with microtubules (not shown) (B) Upon malignant transformation, RHAMM is expressed and exported to the cell surface as monomers and dimers (which from the association from leucine zippers shown in Figure 1).RHAMM surface display of CD44 thereby blocking HA uptake and metabolism. HA fragments accumulate and bind to RHAMM (but not CD44,) The resulting complex enhances surface retention of CD44 hyperactivating CD44-related signaling pathways.

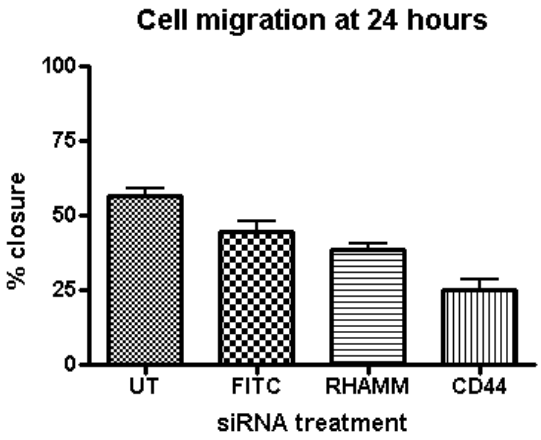
This model has certain predictions that are currently being tested.

1. Co-expression of RHAMM and CD44, as is observed in the highly metastatic PC3M-LN4 cell line, should form a macromolecular complex with ERK 1, 2 that can be detected by co-precipitation. Studies are currently underway to test this model in the prostate cancer model.
2. RHAMM expression should enhance the level of surface CD44 on these cells. The first studies involve using siRNAs against CD44 to limit expression of each and then to evaluate the amount of surface RHAMM remaining following CD44 knockdown. Secondly, RHAMM will be knocked down using RNAi and the level of CD44 remaining will be measured using Western blot and flow cytometry (to determine the surface level of CD44).
3. Inhibiting CD44 expression should limit association of RHAMM with ERK 1, 2 (as assessed by co-precipitation) and the activation state of ERK 1, 2 should be reduced.
4. Finally, limiting HA expression by the PC3M-LN4 cells (which synthesize and capture large amounts of HA on the cell surface) would be predicted to disrupt this complex. This will be accomplished by using hyaluronidase pretreatment or by using RNAi for the hyaluronan synthase enzymes expressed by these cells. The prediction, again, is that limiting HA production will inhibit cell surface RHAMM retention (and possibly inhibit surface levels of CD44).

Time-Line: year 1

Milestones and Outcomes: Evaluation of inhibitory antibodies for CD44 or RHAMM in anchorage independent growth, HA mediated motility/invasion of PC3M-LN4 cells in vitro and subcutaneous tumor growth in xenograft injections

Methods: CD44 and RHAMM expression/function are inhibited both in vitro and in vivo using defined antibodies and specific siRNAs and by stable transfection with conditional expression vectors coding for specific shRNAs. Specific inhibitory antibodies for RHAMM or CD44 are available from both the Turley Laboratory and the Naor Laboratory (see enclosed letter). TET-on conditional anti-CD44 or anti-RHAMM and negative control shRNA expression vectors were purchased from Genscript (Piscataway, NJ). shRNA expression is under the control of the human H1 shRNA promoter containing a tetracycline operator that, in the presence of tetracycline repressor (TetR) inhibits transcription. The vector also contains a coral GFP cDNA under a constitutive promoter, which is used for selecting transfected cells. Initially, tumor cells are transfected with vector pCDNA6/TR (Invitrogen), which constitutively expresses the TetR. Clones that demonstrate high TetR expression are then transfected with the inducible shRNA expression vectors and selected for GFP expression by FACS™. CD44 and RHAMM specific antibodies and siRNAs will be evaluated for their ability to inhibit HA mediated motility and invasion using standard assays available in both the Turley and McCarthy laboratories (time lapse videography, modified Boyden chamber assays). Antibodies will be used to further evaluate the relative contribution of the HA interaction with CD44 or RHAMM in promoting anchorage independent growth in vitro of the metastatic PC3M-LN4 cells. PC3M-LN4 cells stably transfected with conditional shRNA vectors will be utilized to determine the impact of inhibiting CD44 and RHAMM expression on tumorigenic potential by subcutaneous injection or orthotopic injection into the prostate.



Studies have been initiated to determine the effect of inhibiting CD44 expression and function on PC3M-LN4 cell motility (Figure below). The assay used for this experiment is a scratch wound assay in which confluent tumor cell cultures are first treated with CD44 siRNA, RHAMM siRNA, or negative control FITC), incubated for 24 hours, and then the medium is removed and the cultures are wounded with a micropipette tip. The percent closure (relative to the width of the wound at time 0) is calculated from photomicrographs of the assay, thus the smaller the bar, the more effective the inhibitor. The CD44 siRNAs used in this assay effectively and significantly inhibit migration by over 50%, and the knockdown of CD44 protein has been verified (~80%, not shown). The RHAMM siRNA was not significantly inhibitory compared to the FITC control, and analysis of RHAMM protein indicated this siRNA was not effective at inhibiting the level of RHAMM protein. We are in the process of screening additional siRNAs (from Qiagen) for RHAMM to identify those that are effective. The CD44 siRNA treated cells are also in the process of being further characterized for migration and anchorage independent growth.

a. Hyaluronan synthesis and Hyaluronan Synthase expression is being characterized for siRNA treated cells. We have spent the last year generating specific antibodies against specific HAS isoforms. These antibodies have been raised against specific carboxyl terminal regions of all three HAS isoforms. We have synthesized synthetic peptides from these regions and used them to generate polyclonal antibodies in rabbits. These antibodies are currently being characterized by western blots and immunofluorescence in cell and immunohistochemistry of human tissue sections. This will be completed in the next 3-4 months of year 4 and they will be used to evaluate the level of HAS isoforms following siRNA treatment.

b. Expression and Surface Levels of both CD44 and RHAMM are also being analyzed. The prediction is that RHAMM surface levels will decrease with lower levels of surface CD44.We have also contracted for the generation of specific monoclonal antibodies generated against the

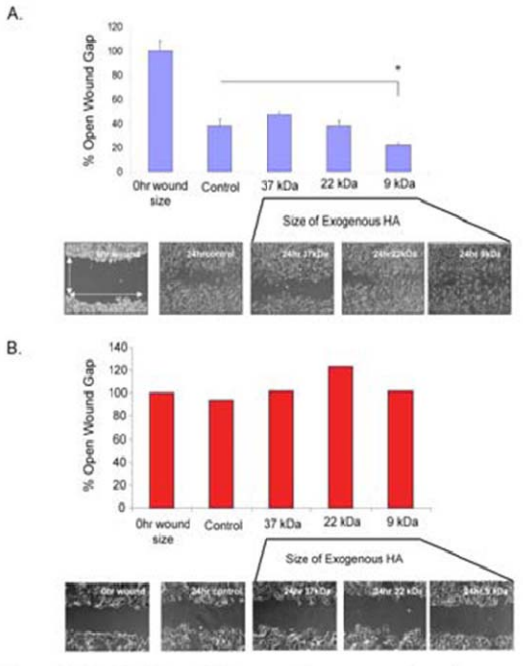
carboxyl terminal coiled coil domain of RHAMM protein. We have received 10 clones that we are also currently characterizing for reactivity against human RHAMM and we will use these antibodies to quantify RHAMM levels on the surface of tumor cells following CD44 siRNA treatment of these tumor cells. The surface level of RHAMM and CD44 will also be evaluated following specific inhibition of HAS isoform expression/HA synthesis by tumor cells. The prediction is that RHAMM levels (and possibly CD44) will decrease following inhibition of HA synthesis in PC3M-LN4 tumor cells.

Task 2: *Evaluate hyaluronan size distribution using FACE analysis of tumors grown as a result of subcutaneous injection of prostate cancer cells.*

Time-Line: year 1

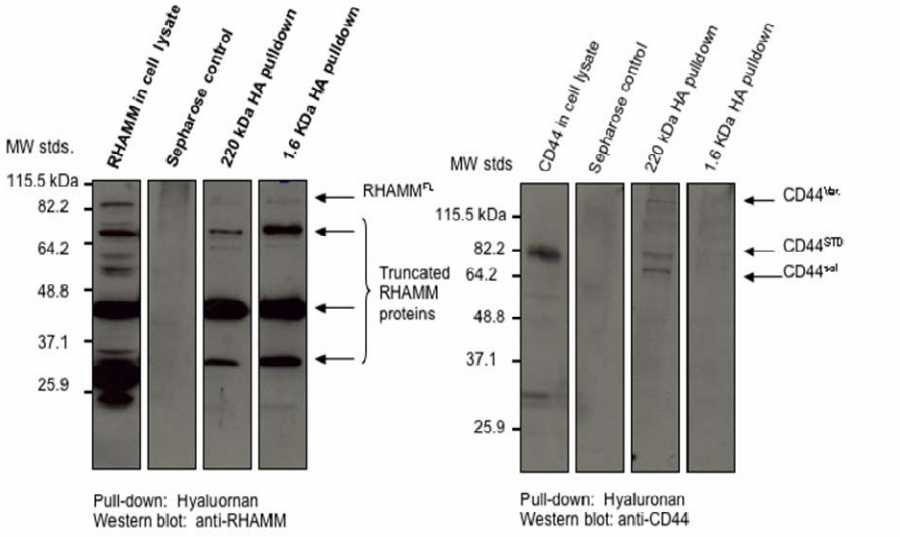
Milestones and Outcomes: Qualitative and quantitative evaluation of HA fragments produced by tumors developed as a result of subcutaneous injection of prostate cancer cells into immuno-compromised mice. The results of this task will determine which size of HA fragments will be used in ITC binding studies.

Methods: Fluorophore-assisted Carbohydrate Electrophoresis (FACE) can be used to determine the amount as well as size of hyaluronan produced in tissues or by cells in culture(Calabro A., et al. Glycobiology 2000; 10: 283-293, Calabro A., et al. Blood. 2002; 100: 2578-2585) and was recently established in the laboratory of E. Turley.



Effect of different HA fragment sizes on wound gap closure by human CaP cells. Migration of serum starved PC3M-LN4 cells (A) or 22RV1 (B) into gaps of scratch wounds. Monolayers were measured in the presence of 37 kDa, 22 kDa, or 9 kDa HA (LifeCore). Wound closure was measured as the size of cell-free wound gap remaining at 24 hours/size of original wound gap. Only 9 kDa HA significantly stimulated migratio PC3M-LN4 cells in this assay. Significant differences are marked by a isks (p<0.01). Values are the Mean and S.E.M (n=3 replicates) in the of PC3M-LN4 and Mean (n=1) alone for 22RV1.

As a first step for this task, hyaluronan oligomers of defined size have been used in assays to define their biological properties. These defined oligomers have been obtained from Seikigaku (Japan) or Life Core (Chaska, MN). The motility promoting activity of these fragments has been evaluated using PC3M-LN4 cells (which express both CD44 and RHAMM) and for 22 RV1 cells (which express low to undetectable levels of both CD44 and RHAMM).The assay used is a scratch wound assay, where, as above, confluent tumor cells in culture are wounded with a pipette, and the closure of these wounds within 24-48 hours is evaluated. The cultures are then photographed and the distance remaining until wound closure is quantified. Data are presented as percent of the gap that remains to be closed by comparing the values with distance at time 0. Thus, in this case larger bars represent slower migration relative to smaller bars. The results of the initial experiments are shown in the Figure above. The hyaluronan oligomers tested ranged from 9kDa to 37kD. Migration was stimulated by the addition of 9kD oligomers compared to that observed in the



Hyaluronan-Sepharose pulls down RHAMM and CD44. PC3LN4 cell lysates were incubated with high molecular HA-sepharose (220kDa) and low molecular weigth HA (960 kDa) columns. Bound RHAMM and CD44 were detected in western blots. Cell lysates were incubated with blocked sepharose columns as controls. RHAMM proteins (full length (FL, 85kDa) and truncated proteins (70, 45, 30 kDa) bound to both high and low MW HA, although more Rhamm protein bound to low MW HA. CD44std (85kDa) variants (120kDa) and soluble (60kDa) bound to high MW HA only but little if any bound to low MW HA. Neither RHAMM nor CD44 protein bound to Sepharose alone. These results are consistent with an ability of High MW HA to "bridge" an association between CD44 and RHAMM.

absence of oligomers, however the larger size HA oligomers failed to stimulate migration over that observed in the control cultures. As expected, the HA oligomers had no detectable effect on the migration of 22RV1 cells.

Our published studies using RHAMM -/- cells and breast cancer cell lines with different levels of cell surface RHAMM indicated that extracellular RHAMM can act as a co-receptor for CD44. Based on these studies, we conclude that these co-receptors can form a signal transduction complex to sustain activation of the ERK 1, 2 pathway. One hypothesis is that different size hyaluronan oligomers will stimulate motility by selectively interacting with cell surface RHAMM. To test this hypothesis, beads coupled to either small (1.6 kDa) or large (220 kDa) hyaluronan oligomers were used to pull down CD44 or RHAMM from the PC3MLN4 cell extracts (Figure above). The results demonstrate that RHAMM interacts with both sizes of hyaluronan oligomers (Left Panel), whereas CD44 only binds to the larger polymer (Right Panel). The results are consistent with a model in which smaller hyaluronan oligomers stimulate motility by selectively binding RHAMM. Since later stage prostate tumors express more RHAMM and increased levels of hyaluronidase, the studies implicate RHAMM in stimulating motility/invasion of later stage prostate tumors.

Our next step in these studies will be to use defined HA fragments to stimulate prostate tumor cells that express CD44 or surface RHAMM. The readouts for these studies include motility and activation of Erk 1,2 pathway and of Akt in the tumor cells. Both of these pathways have been linked to both CD44 and RHAMM stimulation and the prediction is that low molecular weight HA will function to stimulate cells that express cell surface RHAMM (which will be blocked with anti-RHAMM antibodies). In contrast, high molecular weight HA will signal via CD44 and possibly RHAMM (again assessed using specific blocking antibodies to each receptor).

Task 3: *Use ITC with recombinant proteins to characterize binding of hyaluronan oligosaccharides to CD44 and RHAMM to determine if there is a different size requirement for each receptor. Oligosaccharide size will be based on results of task 2.*

Time-Line: years 1 and 2

Milestones and Outcomes: Quantification of binding constants (KDa) of different size HA oligosaccharides to recombinant CD44 and RHAMM protein.

Methods: Isothermal titration calorimetry (ITC) (Pfeil W. and Privalov P.L. Biophys Chem. 1976; 4: 33-40). For this study, HA fragments of specific sizes will be provided by Seikagaku. A buffered solution of hyaluronan fragments will be repeatedly injected (28x10 ul injection, 300-400 sec of spacing) to a buffered solution of recombinant CD44 or RHAMM protein and the energy released as a result of binding between hyaluronan and protein will be measured. Quantification of the released energy will allow calculation of binding constants (KDa).

These studies are not yet started.

Task 4: *Prepare 1st year annual progress report.* Done

Aim 2: ***To identify structural features of CD44 and RHAMM that mediate the effects of hyaluronan on signaling pathways regulating tumor growth, survival and invasion***

Task 1: *Generate mutations of CD44 and RHAMM to interfere with hyaluronan-mediated stimulation of tumor growth, survival and invasion*

Time-Line: Years 1 and 2

Milestones and Outcomes: Site specific mutated constructs of CD44 and RHAMM will be expressed and characterized for the ability to act in a dominant negative fashion to inhibit PC3M-LN4 growth, motility and invasion. Cells that lack or express low levels of CD44 and RHAMM (22Rv1 cells) will be transfected with these mutants to determine if specific mutations fail to mediate hyaluronan stimulated growth or activation of ERK 1, 2.

Methods: We have identified key carboxyl amino acids in murine RHAMM by site-directed mutagenesis that are required for interaction with ERK 1/2 (Zhang et al, 1998) and that are conserved in human RHAMM. We will site mutate full length human RHAMM using the approach we developed for the murine mutant (Yang et al., 1994, EMBO J) and tag the mutated cDNA with a FLAG or HA tag (Zhang et al., 1998). The mutated RHAMM cDNA will be stably expressed in PC3LMLN4 cells. We expect this mutated RHAMM form to act as a dominant negative mutant for RHAMM mediated ERK 1/2 ERK activation by homo-dimerizing with endogenous RHAMM expressed by this cell line, as it does in murine fibroblastic tumors. The effect of the expressed mutated RHAMM on an endogenous RHAMM/ERK co-association will be assessed by co-immunoprecipitating RHAMM/ERK complexes using anti-ERK and conversely anti-RHAMM or anti-tag antibodies. The consequences of expressing mutated RHAMM on ERK activity will be assessed using anti-phospho-ERK antibodies in western analysis of proteins separated from cell lysates using SDS-PAGE (Toelg et al., 2005). A putative docking and phosphoacceptor site at Ser[15] of full length human RHAMM has been identified in Blast programs for conserved sequences. We have obtained preliminary evidence that full length RHAMM is phosphorylated in vitro by ERK 2 kinase. We will site mutate Ser15 to Ala and prepare recombinant RHAMM protein for use as a substrate in an in vitro ERK kinase assay and compare phosphorylation with non-mutated recombinant RHAMM protein. If we verify our preliminary results, we will then stably transfect the 15Ser-Ala mutated RHAMM into both PCLMLN4's, to assess effects as a dominant negative regulator of RHAMM function and 22Rv1 cells to assess growth/tumor promoting effects of this mutated RHAMM form. The consequences of this mutated RHAMM on endogenous ERK activity will be assessed as above. Once these analyses are completed, all stable transfectants produced in the Turley laboratory will be shipped to the McCarthy laboratory for further growth/apoptosis assays.

In Progress. RHAMM expression at high levels by conventional expression vectors is deleterious to cells, requiring the development of alternative expression systems. We have therefore adopted an expression system termed the Rheoswitch inducible expression system, available from New England Biolabs. This system will allow us to precisely induce RHAMM at varying levels of expression in prostate cancer cell lines. The Rheoswitch system consists of two plasmids, the first containing a synthetic nuclear receptor composed of two proteins RheoReceptor-1 and RheoActivator. These two proteins dimerize to form a holoreceptor that blocks transcription of the target gene (in this case RHAMM) in the absence of the synthetic RSL1 ligand. RHAMM will be cloned into the second plasmid and gene expression is controlled by a five tandem repeat of the GAL4 response element. Gene expression is induced by the addition of the synthetic ligand RSL1. RSL1 can be added at a range of concentrations to induce varying levels of gene expression. Cells expressing RheoReceptor-1/RheoActivator are being generated and clones will be selected for those cells that contain high levels of this vector. Selected clones will then be used as recipients for the expression vector containing RHAMM and the cells will be characterized for RHAMM expression in the presence of different levels of the synthetic RSL1 ligand. These cells will be used in migration/growth and invasion assays *in vitro* and in the longer term they will be used in tumor formation assays *in vivo*, since information from New England Biolabs indicates that this expression system can also controlled in vivo by systemic administration of the ligand.

RHAMM variants with the site specific mutations described above are being developed in the Turley laboratory and should be done in the next month 6 weeks. The constructs will then be shipped to the McCarthy laboratory for expression in prostate cancer cell lines that lack RHAMM expression (e.g. 22RV1 cells, shown above), using the Rheoswitch system described above.

We have first characterized the activity of the Rheoswitch system in cells using a GFP vector are in the process of optimizing conditions for the activation of expression in this vector. Rheoswitch vectors will then be constructed using intact RHAMM or specific RHAMM variants for a comparison in prosate cancer cells.

Task 2: *Use epistatic approaches to verify the importance of pErk 1/2 in HA mediated tumor cell growth and motility*

Time Line: Years 1 and 2

Outcomes and Milestones: Stable cell lines expressing constitutively active or dominant negative MEK1 will be generated and characterized for activation of pERK 1/2, anchorage independent growth and HA mediated motility and invasion.

Methods: Conditional expression vectors will be utilized as described above in Task 1/Aim 1. Stable cell lines will be evaluated for activation of ERK 1/2 and for inhibition of anchorage independent growth/tumor formation in mice. Conditionally expressed dominant negative and dominant active MEK1 cell lines will be shipped to the Turley laboratory and the motile response of these cells to HA and defined sizes of HA fragments will be assessed using both Time-lapse cinemicrography (Toelg et al., 2005) and Boyden-style invasion assays using Matrigel.

These studies are not yet started since the RHAMM expression studies have taken priority.

Task 3: *(Year 2). Prepare 2nd year annual progress report.* Done

Aim 3: To test the ability of specific hyaluronan-binding synthetic peptides to inhibit HA binding to CD44 and RHAMM or tumor growth/survival in vitro and in vivo.

Task 1: *Identify which residues of synthetic peptide 15-1 are important for binding HA.*
Time-Line: year 2

Milestones and Outcomes: Identification of amino acid residues responsible for HA binding.

Methods: Screening of a peptide library using biotinylated hyaluronan as probe resulted in the isolation of a 15 mer peptide with partial homology to a 9 amino acid basic motif (BX7B motif) in the hyaluronan binding region of RHAMM. Based on this information we will synthesize peptides in which basic amino acids have been replaced by, for instance, alanine and the binding constant for binding to hyaluronan will be determined by ITC as described under specific aim 1, task 3.

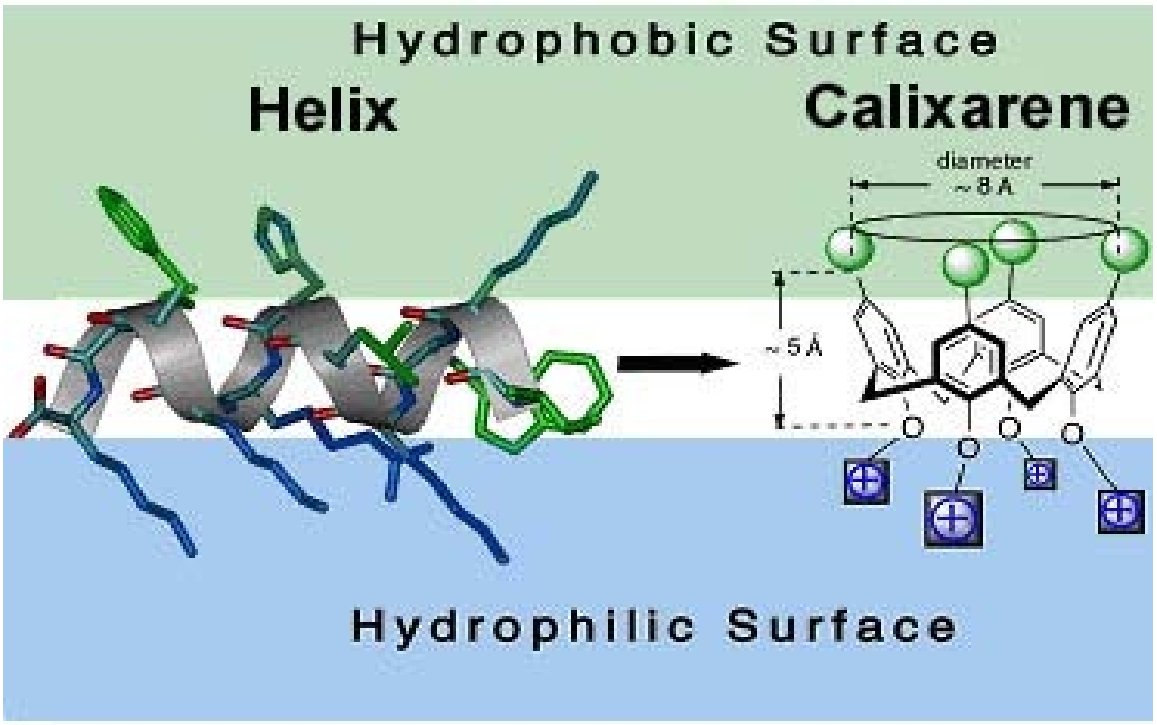
We have established a collaboration with Drs. Mark Klein and Kevin Mayo (University of Minnesota, Dept. Biochemistry, Biophysics and Molecular Biology) who are NMR spectroscopists. These individuals specialize in determining structural/functional properties of peptides and proteins and Dr. Mayo has long standing interest in peptidomimetics/small molecules that inhibit angiogenesis (Dings et al., 2003; Dings et al., 2005; Mayo et al., 2003). Peptide 15-1 (p15-1) is a 15 amino acid peptide [STMMSRSHKTRSHHV] that inhibits HA/receptor interactions. This peptide sequence in the context of its parent protein contains a BX_7B HA binding motif (where B are basic residues and X can be anything except acidic residues) first described in RHAMM, that is also found in many other HA binding proteins (Toole, 2004; Turley et al., 2002). Within the context of its parent peptide, the protein sequence most likely predicts an α-helix motif. However, when the peptide binds HA outside the context of its parent protein, the structural aspects of this interaction are not known with certainty. Structural studies utilizing nuclear magnetic resonance (NMR) techniques are underway to determine the key structural elements in the binding of p15-1 to hyaluronan. NMR can be used to either directly determine a three-dimensional structural model of a peptide, or it can be used to gather evidence for populations of specific structures of that peptide (e.g. α-helix, β-sheet, random coil).

Dr. Klein has recently generated a series of alanine walkthrough variants of peptide p15-1 and we are testing them for the ability to inhibit prostate tumor cell migration. We will use these results in combination with the studies outlined below to determine key residues within peptide 15-1 that are important for HA binding and inhibiting tumor cell migration. Our goal is to use this information for selecting appropriate small molecule libraries to screen for identifying lead compounds. This approach will be performed in parallel with the screening of the calixarene library summarized below. These studies, which are potentially translation in nature, will clearly extend beyond the funding period of the current grant, however studies funded by this grant will be essential for making progress in this area.

The NMR techniques total correlational spectroscopy (TOCSY) and nuclear Overhaüser effect spectroscopy were used to analyze p15-1 in water solution. A combination of TOCSY and NOESY spectra were used to make the initial amino acid assignments. Analysis of the NOESY peaks did not reveal a significant population of peaks corresponding to alpha-helix or beta-sheet (interactions of specific amino residue atoms < 5 Å apart). Subsequently, p15-1 was placed into solution in the presence of sodium dodecyl sulfate (SDS) or dodecylphosphocholine (DPC) to form micelles. It has been shown that micelles can help to stabilize a peptide that contains interconverting structures. Similar experiments did not yield significant NOESY peaks that correspond to α-helix or β-sheet. However, other NMR techniques have revealed evidence of alpha-helical structure of p15-1 in micelles. Determination of the temperature-dependent changes in chemical shift reveals evidence for hydrogen bonding (and therefore, evidence for alpha-helix in this small peptide) if the ratio of the change in chemical shift relative to the change in temperature ($\Delta\delta/\Delta T$) is more positive than -4.5 parts per billion. Data obtained so far have been at two to three points (5, 20, and/or 40 °C) For p15-1 in SDS, $\Delta\delta/\Delta T$ = -3.6 ppb, -3.9 ppb, -2.5 ppb, and -3.4 ppb for residues M4, S5, R6, and H14, respectively. Likewise, for p15-1 in DPC, $\Delta\delta/\Delta T$ = -4, -3.9, and -3.8 ppb for residues S5, S7, and H14. In addition, peptide structure can definitely change upon ligand binding and NMR experiments examining the effect of an hyaluronan octamer binding on the conformation of p15-1 are underway. Any structural differences between the bound and unbound forms of p15-1 would affect the drug design described below.

As part of a collaboration with Dr. Kevin Mayo (U of MN, Biochemistry Dept), we also showed that two α-helix topomimetic compounds based on a calix[4]arene scaffold inhibits the anchorage independent growth of a metastatic hormone independent prostate cancer (cell line PC3M-LN4) at low (micromole) concentrations. This was determined via a screen of a library of these compounds obtained from the Mayo lab. Identifying active compounds in this library would give us several advantages of the use of synthetic peptide, including a lower expense for synthesis and resistance to proteolysis. Calix [4] arenes consist of a cage of 4 benzene rings connected in by intermediary methylene groups (shown in figure below). The diameter of this molecule approximates the diameter of an α-helix or β-sheet, and functional groups can be added to the top or the bottom of the ring to mimic side chains of peptides. The current library of synthesized calixarenes includes 27 variants. The screen referred to above involved 5 representative molecules. Two compounds showed significant inhibitory activity at micromolar concentrations at 7 and 14 days after the start of incubation. Both compounds contain a *tert*-butyl group on each of the benzyl groups on one side of the ring. One of the compounds contains a primary amine connected to each benzyl of the calixarene ring via an ether linkage, and the other consists of a tertiary amine connected to each benzyl via a ester moiety. The other three compounds exhibiting inferior activity differed significantly with respect to the functional groups. These preliminary results suggest different functional groups may be responsible for different calixarene activity in the bioassay. These compounds are currently being analyzed for the ability to inhibit hyaluronan binding to recombinant RHAMM or CD44.

To aid in the design of additional calixarene molecules and to correlate structure and function in an expedited way, we have had 15 peptides synthesized with single site alanine substitutions to determine which residues are important for the binding to hyaluronan in an ELISA assay. A decrease in the binding of a substituted peptide through the use of alanine scanning allows the determination of which residues increase or decrease the binding ability of the peptide relative to the native sequence. The combination of structural and functional properties can be combined to determine the pharmacophore responsible for all or part of a peptide's or small molecule's fundamental activity. The resulting pharmacophore(s) can be overlayed on the calixarene models to design new molecules that mimic the peptide, but are likely to be resistant to proteolysis. Other substitutions can be made that potentially could increase the binding of the peptide relative to the native protein for the design of similar molecules.



Task 2: *Evaluate peptide 15-1 for inhibiting hyaluronan binding by CD44 and RHAMM*
Time-Line: Year 2.

Milestones and Outcomes: Complete studies to determine effectiveness of peptide 15-1 to compete for binding to RHAMM or CD44.

Methods: Peptide 15-1 will be used in competition assays using ELISA type assays of hyaluronan binding to immobilized recombinant RHAMM or CD44.

These studies have not yet been started. The emphasis has first been placed on structural/functional studies described above.

Task 3: *Determine efficacy of peptide 15-1 for inhibiting growth, motility and invasion of prostate carcinoma cells in vitro*

Time-Line: Years 2 and 3.

Milestones and Outcomes: Complete the in vitro analyses of the inhibitory effects of peptide 15-1 in anchorage independent growth, motility and invasion in vitro.

Methods: Peptide 15-1 or appropriate control peptides will be mixed with PC3M-LN4 cells and tested for the ability to inhibit growth in methyl cellulose. Cells preteated with peptide will also be tested for the ability to activate pERK 1/2 in response to serum, growth factors or hyaluronan. Motility/invasion will be analyzed using both Time-lapse cinemicrography (Toelg et al., 2005) and Boyden-style invasion assays using Matrigel.

These studies have not yet been started. The emphasis has first been placed on structural/functional studies described above.

Task 4*: Evaluate peptide 15-1 for inhibiting tumor growth in orthotopic and intrafemoral injection xenograft injection models for human prostate cancer.*

Time-Line: Years 2 and 3.

Milestones and Outcomes: Complete analysis of inhibitory effect of peptide 15-1 on tumor growth in tumors injected via an orthotopic or intrafemoral route

Methods: Cells will be mixed with peptide and injected into animals either orthotopically or intrafemorally. At the conclusion of the assay, the animals will be sacrificed, the tumors excised from the prostate and weighed/sectioned for histological analysis. Animals in which tumors have been injected into bone (or sham injected) will be X-rayed to estimate bone density and animals will be sacrificed, bones will be harvested and processed for histology. Histological analysis includes estimation of vascularization and hyaluronan content using specific probes available in the McCarthy laboratory.

These studies have not yet been started. The emphasis has first been placed on structural/functional studies described above.

Task 5: (Year 3) Prepare 3rd year annual progress report. This is the current report.

Key Research Accomplishments:

1. Demonstration that cell surface RHAMM has functional significance in wound microenvironments in vivo.

2. Cell surface expression of RHAMM causes sustained activation of the ERK 1, 2 pathway.

3. RHAMM stimulated motility requires CD44 expression

4. Demonstration that cell surface RHAMM forms complexes with CD44 in tumor cells which interact with elements of the ERK 1, 2 pathway.

5. Demonstration that hyaluronan stimulated motility of prostate tumor cells depends on the size of the HA oligomers. Smaller hyaluronan oligomers are more effective than larger oligomers.

6. Demonstration that RHAMM selectively interacts with smaller hyaluronan oligomers, whereas CD44 binds to larger polymers.

7. Generated antibodies against specific HAS isoforms and RHAMM to be used to further evaluate the model proposed above. The antibodies are currently being characterized for reactivity and specificity and will be used in studies to be done during the upcoming year.

8.

Reportable Outcomes:

McCarthy, J.B. and E.A. Turley. 2009. RHAMM/HMMR: An Itinerant and Multifunctional Hyaluronan Binding Protein that Modifies CD44 Signaling and Mitotic Spindle Formation Seminars in Cancer Biology. In Press.

Conclusion:

Progress has been made on this funded proposal to identify mechanisms by which tumor associated hyaluronan can facilitate tumor growth and invasion. Analysis of human tissue microarrays shows a clear association of increased levels of RHAMM, hyaluronan and hyaluronidase expression. Our studies from two other systems (included in Appendix) have clarified a role for extracellular RHAMM in mediating motility and activation of the ERK 1, 2 pathway both in vivo and in vitro. Furthermore, these studies have demonstrated that RHAMM acts a co-receptor for CD44 in invasive/metastatic breast cancer tumor cell lines and these studies serve as a model to be tested in advanced prostate cancer cell lines used for this study. Furthermore, fragmented hyaluronan was shown to be more effective than higher molecular weight oligomers at binding RHAMM and stimulating prostate tumor cell motility. These studies are consistent with a model in which fragmented HA and RHAMM interact in more advanced tumor cells to stimulate motility and invasion. Finally, a synthetic hyaluronan binding peptide, which was shown in preliminary data in the proposal to inhibit tumor formation in vivo and anchorage independent growth in vivo, has been partially characterized using NMR. The results suggest that the peptide has an interconverting α-helical structure and additional studies in the presence of hyaluronan are being initiated to determine if the interaction stabilizes the conformation of the protein. The goal of these studies is to determine the active residues within the peptide that are important for hyaluronan binding, with the hope that such information could provide a rationale for selecting a small molecule library to further interrogate. Studies using a small molecule library which has properties that would be predicted to be important for binding to hyaluronan have also been started as a parallel approach for designing compounds that will inhibit hyaluronan/tumor cell interactions and reduce growth, motility, and possibly resistance to therapies currently in use.

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Appendices:

1.) **McCarthy, J.B. and E.A. Turley. 2009.** RHAMM/HMMR: An Itinerant and Multifunctional Hyaluronan Binding Protein that Modifies CD44 Signaling and Mitotic Spindle Formation. Seminars in Cancer Biology. In Press.

CHAPTER

9

RHAMM/HMMR: An Itinerant and Multifunctional Hyaluronan Binding Protein that Modifies CD44 Signaling and Mitotic Spindle Formation

James B. McCarthy and Eva A. Turley

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OUTLINE

INTRODUCTION

p0015 RHAMM was originally isolated as a secreted fibroblast protein in a model of wounding *in culture* (Fig. 9.1) (Turley, 1982). In this study, embryonic heart tissue was explanted and the supernatant medium was

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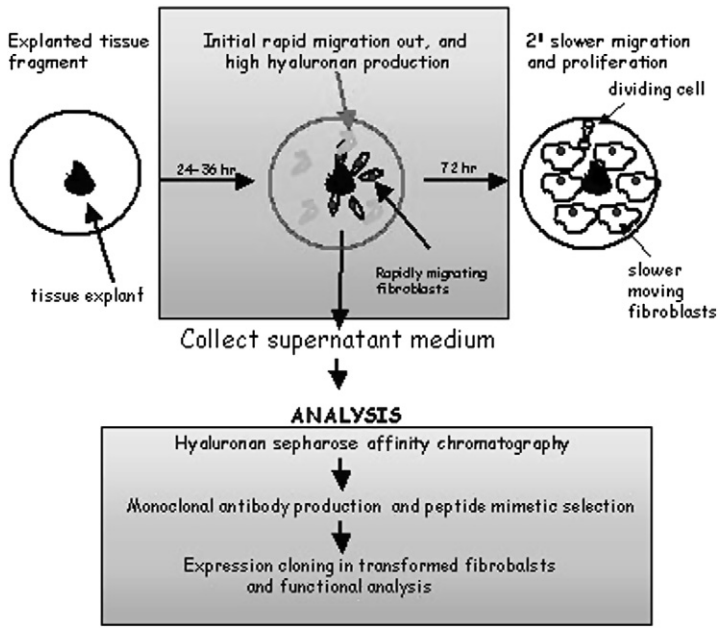
collected for further analysis between 18 and 24 hours after explantation, when fibroblasts were rapidly migrating and producing high levels of HA. Sampling at this time excluded analysis of dividing cells and interphase cells that had formed actin stress fibers/focal contacts, since these events do not occur until approximately 36 hours after explantation. Hyaluronan binding proteins were isolated using hyaluronan Sepharose chromatography, analyzed on SDS-PAGE, and purified proteins were extracted and used for preparation of polyclonal and monoclonal antibodies (Turley et al., 1985; 1987). Expression cloning using these reagents resulted in the isolation of RHAMM (Hardwick et al., 1992). RHAMM is a coiled coil protein that first appeared in vertebrates and is not found in lower organisms or in insects (Fig. 9.2).

p0020 Given its roles in such important cellular processes as motility and cell division *in culture* (Turley et al., 2002; Slevin et al., 2007), it is surprising that germline genetic deletion of RHAMM does not result in a detectable phenotype during embryogenesis or adult homeostasis (Tolg et al., 2003; 2006). However, loss of RHAMM does affects the tissue response to injuries



F0010 **FIGURE 9.1** Schemata used to isolate secreted RHAMM from rapidly migrating fibroblasts *in culture*. Embryonic chick hearts were explanted and fibroblasts allowed to rapidly migrate out of the explants for 24–36 hours. Cell division and a secondary stage of slower cell migration does not occur until 72 hours. The supernatant medium was decanted and hyaluronan binding proteins were captured using hyaluronan-Sepharose affinity columns. Monoclonal antibodies were prepared against the captured proteins and these were used for expression cloning (Turley, 1982).

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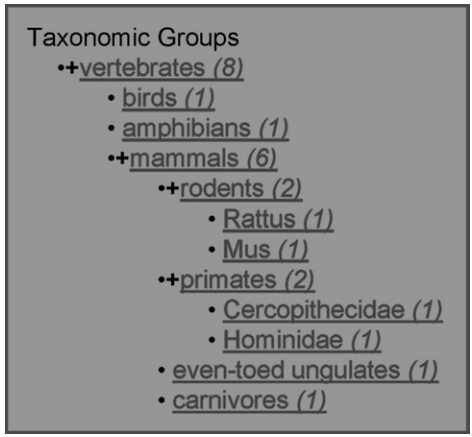

FIGURE 9.2 Taxonomic groups in which RHAMM has emerged. RHAMM first appears in vertebrates and has been identified in mammals, birds and amphibians. No structural orthologues have yet been identified in lower organisms.

including excisional cutaneous wound repair and bleomyocin-induced lung damage (Tolg et al., 2006; Slevin et al., 2007). Excisional cutaneous wounds repair occurs via several well-regulated stages that include initial inflammation, proliferation and remodeling (Dorsett-Martin, 2004; Gibran et al., 2007; Oberyszyn, 2007). The inflammatory and proliferative (fibrogenesis) stages are subtly altered by loss of RHAMM (Tolg et al., 2006). For example, neutrophil accumulation remains high throughout the inflammatory and post-inflammatory stages of wound repair in RHAMM$^{-/-}$ mice whereas these cells have undergone apoptosis within several days following injury in wild-type animals. Fibrogenesis of RHAMM$^{-/-}$ wounds is aberrantly decreased due to reduced fibroblast infiltration compared to fibrogenesis/fibroblast infiltration in wild-type animals. Mesenchymal differentiation during fibrogenesis is also modified and/or inappropriately regulated. For example, granulation tissue of RHAMM$^{-/-}$ wounds is filled with adipocytes and Rhamm$^{-/-}$ fibroblasts explanted from wounds undergo adipogenesis *in culture* to a much greater extent than wild-type wound fibroblasts. Myofibroblast activity is reduced in Rhamm$^{-/-}$ wounds, as reflected by the relative lack of differentiation markers such as smooth muscle actin. Furthermore, muscle differentiation within the wound is impaired (Tolg et al., 2006). The many similarities between responses to tissue injury and cancer suggest that RHAMM may be important in the progression of malignant tumors.

S0015 RHAMM AND CANCER

p0025 Although RHAMM mRNA is rarely detected in most homeostatic adult tissues, it is commonly hyper-expressed in human cancers. Cell surface RHAMM has been detected as a tumor antigen in several malignancies,

including leukemia, breast, melanoma, prostate and ovarian cancers (Greiner et al., 2006; Schmitt et al., 2008). Intracellular RHAMM expression is also elevated above normal tissue levels in brain tumors (e.g. astrocytomas, gliomas, meningiomas) (Panagopoulos et al., 2008), gastric cancer (Li et al., 2000a), colorectal cancer (Zlobec et al., 2008), multiple myeloma (Maxwell et al., 2004), oral squamous cell carcinoma (Yamano et al., 2008), endometrial carcinomas (Rein et al., 2003) and bladder cancer (Kong et al., 2003). RHAMM is often most highly expressed in carcinomas at later stages of progression and a relationship between RHAMM hyper--expression and the levels of RHAMM have been correlated to tumor stage and in some cases to prognosis. For example, RHAMM hyper-expression is an independent prognostic indicator of poor clinical outcome in breast cancer (Wang et al., 1998; Pujana et al., 2007), colorectal cancer (Zlobec et al., 2008), multiple myeloma (Maxwell et al., 2004), and oral squamous cell carcinoma (Yamano et al., 2008).

p0030 The prognostic value of RHAMM is linked to specific subtypes of organ specific malignancies, which have distinct anatomic and molecular pathologies that are correlated to different outcomes. For example, colorectal cancers can be grouped into DNA mismatch repair-proficient, MLH1 negative and presumed Lynch syndrome. Although RHAMM is expressed at all Dukes stages of ungrouped colorectal cancers (Yamada et al., 1999), hyperexpression of RHAMM mRNA is an independent prognostic factor for poor outcome and ranks higher than T stage, vascular invasion, tumor budding and tumor grade in its association with increased risk of peripheral metastasis and with worse outcome in patients with metastatic disease (Zlobec et al., 2008a, b). Additional analysis reveals that RHAMM is a prognostic factor in DNA-mismatch repair-proficient (MMR-proficient) and presumed Lynch syndrome forms of colorectal cancer but not in MLH1 negative colorectal tumors (Duval et al., 2001; Lugli et al., 2006; Zlobec et al., 2008c). Furthermore, combining RHAMM hyper-expression with another prognostic factor, loss of p21, identifies a subgroup of MMR-proficient tumors with a high incidence of microsatellite instability that has a particularly poor prognosis (Zlobec et al., 2008c). These results predict that the association of elevated RHAMM expression with neoplastic disease may be selective in terms of tumor subtype. It may therefore be possible to use RHAMM levels as one marker to further classify specific tumors or to target RHAMM for therapy in such tumor subtypes.

p0035 In addition to increased levels of expression, distinct RHAMM isoforms that arise through multiple mechanisms are associated with tumors and some of these isoforms appear to behave as multifunctional oncogenes. Two major alternatively spliced mRNA species have been identified, which differ in the presence of exon 4, (which encodes a 15 amino acid sequence in the amino terminal region of RHAMM). For example, $Rhamm^{-exon4}$ is

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expressed by aggressive multiple myeloma subsets (Maxwell et al., 2004). Other tumor types (including breast carcinoma, astrocytoma, multiple myeloma, melanoma, gastric carcinoma and astrocytomas), which have been reported to hyperexpress RHAMM mRNA also express truncated RHAMM protein forms (Li et al., 2000a, b; Ahrens et al., 2001; Zhou et al., 2002; Hamilton et al., 2007). Although the basis for the formation of these smaller proteins (which range from 40 to 70 kD compared to 86 kD full length Rhamm protein) is not known, it is possible that they arise as a result of partial proteolytic processing and/or the use of multiple start codons, which are present within the coding sequence of RHAMM. Human astrocytomas express a 70 kD tumor specific RHAMM isoform in addition to the full length protein (86 kDa) which is expressed in low amounts by normal astrocytes (Zhou et al., 2002). Gastric carcinomas, breast carcinomas and melanomas also express multiple RHAMM isoforms (52–70 kDa) in addition to the full-length form (Li et al., 2000b; Ahrens et al., 2001; Hamilton et al., 2007). Highly invasive human breast cancer cell lines such as MDA-MB-231 similarly express a 70 and 52 kD Rhamm protein compared to less aggressive lines such as MCF7, which primarily express the full length RHAMM protein (Hamilton et al., 2007). It is also possible that some of these additional lower molecular weight isoforms are created from low abundance splice variants of RHAMM mRNA, however no such variants have been identified to date.

p0040 Several studies also suggest that mutations in RHAMM can confer oncogenic properties. RHAMM contains mononucleotide repeat coding sequences that are mutated in human MMR type colorectal cancers with high microsatellite instability (Duval et al., 2001). These mutations result in conversion of intronic 3′ non-coding to a coding sequence which creates additional protein sequence resembling the HA binding region of RHAMM. Malignant peripheral nerve sheath tumors arising from neurofibromatosis type 1 (risk is 10%) often exhibit an allelic (N = 1) deletion of a RHAMM gene (Levy et al., 2004; Mantripragada et al., 2008). Germ line polymorphisms upstream of the coding sequence in the RHAMM gene are also associated with a predisposition to breast cancer suggesting that mutated RHAMM may function as a novel breast cancer susceptibility gene (Pujana et al., 2007).

p0045 Finally, one of the more intriguing changes in RHAMM that is associated with oncogenesis is the change in both the subcellular distribution and functions of the protein (Maxwell et al., 2008). In examples such as gastric and highly malignant breast cancers, RHAMM is exported to the cell surface where it functions as a hyaluronan-responsive motogenic protein (Li et al., 2000a; Hamilton et al., 2007). Extracellular RHAMM is detected on a number of tumor cell lines cultured *in vitro*. In this case, cell surface RHAMM is more pronounced in subconfluent, rather than confluent cultures (Turley, 1982; Hardwick et al., 1992) and the addition of hyaluronan

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acts to further increase the amount of cell surface RHAMM (Gao et al., 2008). As has been observed with many other non-conventionally exported proteins such as autocrine motility factor and galectins 1 and 3 (Radisky et al., 2003; Nickel, 2005; Prudovsky et al., 2008), extracellular RHAMM functions, at least in part, to enhance motility as well as invasion of tumor cells (Hamilton et al., 2007). However, in certain tumors such as mesenchymal desmoid tumors, alterations in RHAMM may function to promote both tumor initiation and as invasion (Tolg et al., 2003). RHAMM is highly expressed in human mesenchymal desmoid tumors (fibromatoses). In an animal model of desmoid tumor susceptibility, both the number (which equates to initiation) and size (which equates to growth and invasiveness) of tumors are decreased when RHAMM is germ line deleted as a result of homologous recombination (Tolg et al., 2003).

p0050 Although the molecular mechanisms for the unconventional export and other subcellular changes in the distribution of RHAMM during tumorigenesis are not yet known, similar changes occur during normal wound healing and tissue repair (Zaman et al., 2005; Tolg et al., 2006; Slevin et al., 2007), which are processes that- require cellular infiltration and remodeling of the microenvironment. Thus, some of the functions of extracellular RHAMM in tumors may mirror those observed in wound healing, which like tumor progression requires both cellular infiltration and tissue remodeling.

S0020 EXTRACELLULAR AND INTRACELLULAR RHAMM ONCOGENIC FUNCTIONS *IN CULTURE*

p0055 RHAMM was originally identified as a ''secreted'' hyaluronan binding protein that was isolated from the supernatant medium of rapidly locomoting, sparsely cultured chick embryonic fibroblast (Turley, 1982). The binding of purified, secreted RHAMM protein to fibroblast monolayers is both saturable and of high affinity (Turley et al., 1985). These and other data led to the prediction that secreted RHAMM acts as a co-receptor that associates with binding sites or other receptors located at the cell surface (Hardwick et al., 1992). More recently, cell surface RHAMM has been shown to function as a motility receptor in both dermal fibroblasts responding to injury and in aggressively invasive breast cancer cell lines that have undergone EMT by forming complexes with CD44 (Tolg et al., 2006; Hamilton et al., 2007). RHAMM coated beads were used to demonstrate that RHAMM interactions with the cell surface enhances the cell surface display of CD44. Additional studies using RHAMM$^{-/-}$ fibroblasts have shown that RHAMM-stimulated motility is sensitive to antibodies against CD44 and that RHAMM stimulates Erk1,2 activity through its interaction with CD44 (Tolg et al., 2006). These and other data (summarized below) indicate that

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cell surface RHAMM functions as a co-receptor for CD44 in wound fibroblasts and in breast cancer cells that have undergone an EMT (e.g. MDA-MB-231 cells) (Tolg et al., 2006; Hamilton et al., 2007). Furthermore, this interaction is necessary for sustained activation of motogenic signaling pathways through ERK1,2. Cell surface RHAMM also associates with RON (gene name, MST-1R), a tyrosine receptor kinase of the c-Met family, whose ligand is macrophage stimulating protein (MSP) (Manzanares et al., 2007). Thus extracellular RHAMM functions as a co-receptor with one or more adhesion/growth factor receptors on the cell surface, with the ability to alter the intensity and/or duration of key signal transduction pathways.

p0060 Extracellular export of RHAMM may be particularly relevant to tumor therapy. For example, patients with certain malignant tumors were shown to have increased levels of RHAMM in their circulation predicting that this protein might be used as a tumor specific marker (Greiner et al., 2006). Indeed, dendritic vaccination using RHAMM peptides has shown efficacy in phase I clinical trials of patients with acute myeloid leukemia (AML) and multiple myeloma (Schmitt et al., 2008). Furthermore, vaccination of mice using dendritic cells transfected with RHAMM mRNA induces an anti-tumor effect associated with increased activation of the immune system in a model of mouse glioma (Amano et al., 2007). Thus, tumors may be susceptible to these and other RHAMM-targeted therapies as a result of the increased levels of cell surface protein expressed on these tumors.

p0065 While initial reports on the function of RHAMM focused on its extracellular functions, the cloning and further characterization of RHAMM primary structure revealed the protein lacks a signal peptide for classical secretion through the Golgi/ER or a membrane spanning sequence typical of traditional transmembrane receptors (e.g. CD44) (Maxwell et al., 2008). Several subsequent studies therefore focused on identifying the functions of intracellular RHAMM protein forms, since its sequence was more in line with that of a cytoplasmic or nuclear protein. These studies demonstrated that intracellular RHAMM is associated with interphase microtubules, it can be localized to the nucleus (Hofmann et al., 1998a; Liska et al., 2004; Shakib et al., 2005), and it also co-distributes with centrosomes/mitotic spindles (Maxwell et al., 2003; Evanko et al., 2004). This complex subcellular distribution of RHAMM in tumor cells has created challenges for developing models to study the importance of cell surface vs. intracellular RHAMM.

p0070 The addition of soluble, recombinant extracellular RHAMM to Ras-transformed fibroblasts was originally observed to inhibit TGF-β induced progression through the cell cycle by inhibiting cyclin B1 and cdc2 expression thus restricting passage through G2/M (Mohapatra et al., 1996). Additionally, it has been shown using an unbiased expression array screen in synchronized, proliferating cells that RHAMM mRNA levels peak at or near the G2/M boundary of the cell cycle (Cho et al., 2001;

Whitfield et al., 2002; Groen et al., 2004; Liska et al., 2004; Yang et al., 2005) and RHAMM is one of the numerous mitotic spindle proteins that are phosphorylated during spindle formation (Nousiainen et al., 2006). Furthermore, intracellular RHAMM is required for progression of certain cell types through G2/M (Maxwell et al., 2003). Collectively these studies imply that control of G2/M by RHAMM may be coordinated by both extracellular and intracellular forms of this protein. However, the precise relationship between the mitotic functions of these two pools of RHAMM has not yet been established.

p0075 Cells derived from RHAMM$^{-/-}$ animals provide an important tool to address this problem. Studies using recombinant RHAMM-coated beads to challenge RHAMM$^{-/-}$ fibroblasts has shown that these beads are sufficient to stimulate the motility of these cells over 4 hours indicating that cell surface RHAMM alone is sufficient to promote short spurts of motility (Tolg et al., 2006). On the other hand, recombinant RHAMM coated beads do not rescue the aberrant mitosis of RHAMM$^{-/-}$ fibroblasts (Tolg et al., 2006). Collectively, the evidence indicates that intracellular RHAMM protein levels (e.g. too much or too little) are necessary and sufficient to control mitotic spindle formation and stability. More recently, the spindle formation function of RHAMM has been shown to be part of the BRCA/BARD1 E3 ubiquitin ligase pathway (Pujana et al., 2007; Joukov et al., 2006). This pathway has the potential for promoting genomic instability in tumors in which BRCA1 is compromised by mutation. The mitotic spindle functions of RHAMM may also, at least in part, explain the importance of RHAMM as a novel breast tumor susceptibility gene.

p0080 These motogenic and mitogenic effects of RHAMM have been demonstrated *in culture*, and although they have the potential to contribute to cancer initiation and progression is there any evidence that RHAMM performs similar motogenic and mitogenic functions *in vivo*? As noted above, RHAMM polymorphisms and hyper-expression have been linked to cancer susceptibility and poor clinical outcome in a number of human cancers, however analyses of RHAMM gene dysregulation using animal models has not been extensively reported. To date, an *in vivo* analysis of the mitogenic and motogenic effects of RHAMM have been reported in a mouse model of collagen-induced arthritis (Nedvetski et al., 2004), during lung repair of bleomycin induce injury (Zaman et al., 2003), during repair of full thickness, excisional skin wounds in RHAMM$^{-/-}$ mice (Tolg et al., 2006), and in desmoids/upper intestinal tract tumor formation in a transgenic mouse model of these tumors (Tolg et al., 2003).

s0025 Functions of RHAMM in Animal Models of Repair and Disease

p0085 Collagen-induced arthritis in both wildtype and CD44$^{-/-}$ mice is dependent upon the production of hyaluronan since destruction of this

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polysaccharide by hyaluronidase injected into the toe joint blocks its development (Naor et al., 2007). In wild-type mice, blocking CD44 function also stops the appearance of arthritis in joints but genetic loss of CD44 intensifies rather that abrogates arthritis development. In this model, extracellular RHAMM compensates for CD44/hyaluronan binding function since injection of anti-RHAMM antibodies or recombinant RHAMM reduces the inflammatory response in the absence of CD44 (Nedvetski et al., 2004). However, compensation is accompanied by an intensified disease course due to the up-regulation of pro-inflammatory cytokines that are highly expressed in the absence of CD44. The molecular mechanisms by which RHAMM affects inflammation in this model have not yet been not identified but the process is associated with increased accumulation of hyaluronan in the toe joints of $CD44^{-/-}$ animals, which acts to promote signaling through cell surface RHAMM. Although RHAMM partners with CD44 in some systems (see below), this is not possible in the $CD44^{-/-}$ mice, suggesting that cell surface RHAMM may partner with an as yet unidentified integral receptor on spleenocytes (Nedvetski et al., 2004).

p0090 Macrophage infiltration into injured lung tissues also requires cell surface expression of RHAMM (Zaman et al., 2005). In this model of lung injury, TGF*B*1 production is stimulated by bleomycin and this cytokine promotes expression of cell surface RHAMM and motogenic influx of macrophages to the site of injury. Although macrophages express both cell surface RHAMM and CD44, blocking the motogenic function of cell surface RHAMM with anti-RHAMM antibodies alone reduced macrophage influx and the concomitant fibrotic response resulting from bleomycin. Collectively, these two studies using injury models demonstrate a role for cell surface RHAMM in controlling influx of inflammatory cells into wounded tissues thereby affecting the course of tissue repair.

p0095 Germline genetic deletion of RHAMM results in the wound mesenchymal defects noted above (Tolg et al., 2006). In $RHAMM^{-/-}$ mice, reduced fibroplasia is related to a motogenic defect associated with reduced responsiveness *in culture* to motility-promoting stimuli such as PDGFBB and hyaluronan oligosaccharides (Tolg et al., 2006). Surprisingly, neither increased apoptosis nor reduced proliferation is detected in the wound site although markers for motility are aberrantly expressed. A motogenic defect of $RHAMM^{-/-}$ fibroblasts is retained *in culture* and results from reduced display of cell surface CD44 and aberrant activation kinetics of ERK1,2. This study also demonstrated that CD44 and RHAMM co-associate to regulate the duration of ERK1,2 motogenic signaling during tissue repair. Furthermore, the motogenic defect of $RHAMM^{-/-}$ wound fibroblasts can be rescued either by the stable expression of constitutively active MEK1 or by addition of extracellular recombinant RHAMM, which was prevented from intracellular uptake by linkage to Sepharose beads (Fig. 9.3).

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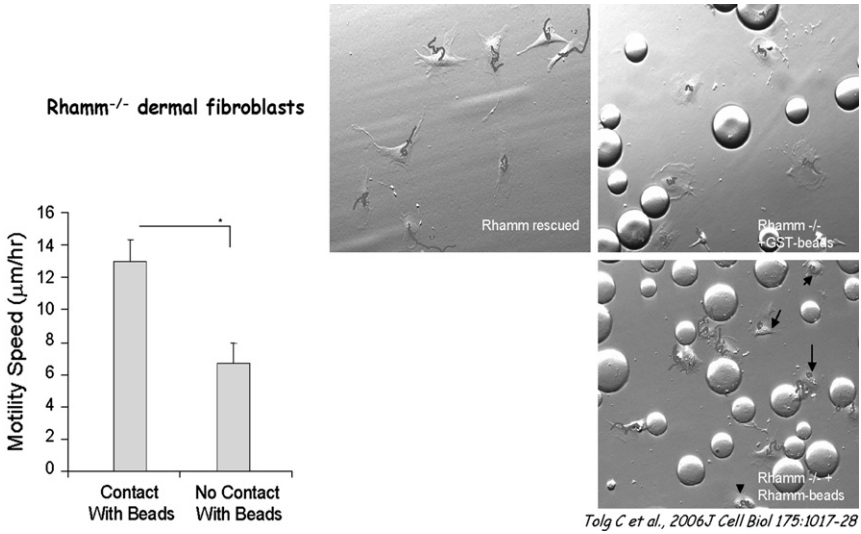

F0020 **FIGURE 9.3** Cell surface but not intracellular RHAMM is required for acute migration in response to hyaluronan or growth factors. RHAMM$^{-/-}$ dermal fibroblasts were exposed to recombinant GST-RHAMM protein linked to Sepharose beads or to GST alone. GST-RHAMM-beads that touch RHAMM$^{-/-}$ fibroblasts promote migration while GST-beads or untouched beads (detected by real time analysis) do not. Since cells cannot internalize beads coated with recombinant RHAMM, showing that cell surface RHAMM is sufficient to promote migration, at least over the 4 h assay period. From Tolg et al. (2006).

p0100 Two studies have also investigated the roles of RHAMM in facilitating the growth of tumors *in vivo*. In a xenograft injection model using MDA-435 tumor cells, expression of the hyaluronan binding region of RHAMM (Liu et al., 2004) results in decreased growth rate compared to control tumor cells lacking this peptide. The slower growth produced by the expression of the hyaluronan binding domain of RHAMM results from the ability of this peptide to activate caspase 3 and 8 as well as poly-(ADP-ribose) polymerase, thereby promoting apoptosis of the transfected tumor cells. The effect of RHAMM expression in the initiation and growth of desmoid tumors in a syngeneic mouse environment has also been evaluated (Tolg et al., 2003). For these studies, RHAMM$^{-/-}$ mice were crossed to Apc/Apc1638N transgenic mice (which are predisposed to desmoids (fibromatoses) and upper intestinal tract tumors). RHAMM deficiency significantly reduces both the number (equated to initiation) and size (equated to invasion) of desmoids but not upper intestinal tract tumors. These effects of RHAMM on tumorigenesis are apparently related to a desmoid tumor cell proliferation deficiency that is observed at low but not high cell density *in culture*. Currently therefore, these *in vivo* data support a role for RHAMM in both cell motility and proliferation and

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further suggest a potential role in apoptosis. Is there any clinical evidence that RHAMM performs similar functions in human cancers?

s0030 Functions of RHAMM in Human Tumors

p0105 The selectively high expression of RHAMM in tumor cell subsets (Wang et al., 1998; Pujana et al., 2007) and at the invading front of primary breast cancers (Assmann et al., 1998) is consistent with the ability of RHAMM to promote motogenic/invasive activities of tumors that have been identified *in culture*. Proteomics and integrative genomics of human cancers also point to a role for RHAMM in mitosis. For example, DNA microarray analyses have revealed that RHAMM expression is cell cycle G2/M regulated in hepatic carcinoma (Yang et al., 2005) and RHAMM expression is correlated with centrosomal structural abnormalities in multiple myeloma (Maxwell et al., 2005). These studies are consistent with evidence indicating that intracellular RHAMM has a mitotic spindle/centrosomal function *in culture*. But how do the multiple RHAMM protein forms control these disparate cellular functions?

S0035 RHAMM PROTEIN FORMS CONTROL MULTIPLE SIGNALING NETWORKS

p0110 Neither structural nor phylogenetic analyses of RHAMM have provided clues as to the molecular mechanisms by which the protein products of this gene influence cell motility and cell division. The secondary structure of RHAMM is largely a hydrophilic coiled coil (Turley et al., 2002; Groen et al., 2004), with the exception of an N-terminal 162 amino acid globular region (encoded by exons 1–4) and additional interspersed linker regions connecting the helical domains of the coiled coils (Fig. 9.4). These analyses suggest intracellular RHAMM performs mainly structural functions yet the majority of experimental data point to the importance of this protein in activation of signal transduction pathways. RHAMM primary sequence contains neither a signal peptide nor a membrane spanning sequence that would predict its ability to be exported from the cell or to activate signaling cascades once it is exported yet again evidence *in vitro* shows a clear role for extracellular RHAMM in motogenic signaling (Hofmann et al., 1998b; Maxwell et al., 2008), Furthermore, although it contains putative nuclear localization and export sequences (and is found in the cell nucleus), it does not contain sequences that predict its localization to the mitotic spindle, mitochondria, interphase microtubules or actin filaments. These results suggest that interaction of RHAMM with these subcellular components is either indirect or involves unique structural domains that mediate binding.

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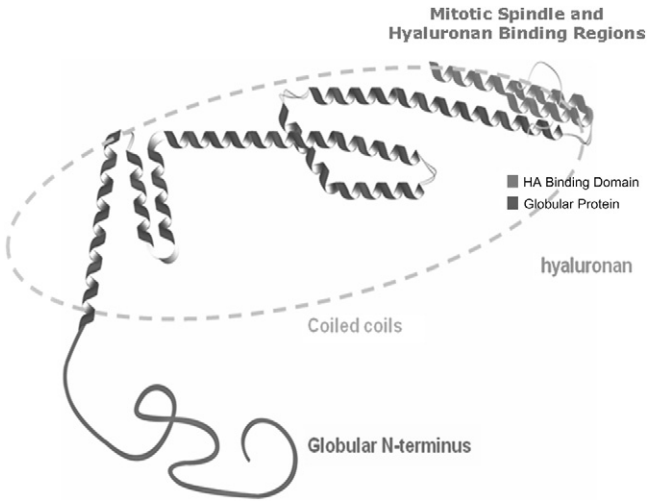

F0025 **FIGURE 9.4** The predicted secondary structure of RHAMM. RHAMM is largely an acidic coiled coil protein. Approximately $^{3}/_{4}$ of its sequence occurs as a coiled coil similar to TACC, myosin and other coiled coil proteins. The N-terminal 163 amino acids form a globular peptide. The mitotic spindle binding (a leucine zipper) and hyaluronan binding (highly basic region) are localized to the carboxyl terminus of RHAMM.

p0115 Phylogenetic analysis of RHAMM sequence has also not clarified the molecular mechanisms by which RHAMM could affect such basic cell functions as motility and mitosis. RHAMM orthologs first appeared in vertebrates but there is no significant homology to proteins in lower organisms such as *Drosophila*, worms or yeast that might provide hints as to the functions of this gene (Fig. 9.2). Indeed, a report suggesting the RHAMM represents the mammalian TACC4 gene (Maxwell et al., 2003), (a gene found in lower organisms that encodes for a protein structurally similar to RHAMM) (Still et al., 2004). As a result, a conundrum has arisen surrounding not only the motogenic and mitogenic functions of RHAMM protein forms but also when and where these functions come into play. On the one hand experimental (but not proteomic) analysis of RHAMM shows it performs essential motogenic and cellular functions as a cell surface co-receptor and mitogenic functions as a mitotic spindle binding protein *in culture* and during tissue repair *in vivo*. The conundrum then is this: How can a cell surface co-receptor also function as a mitotic spindle protein?

p0120 Growing evidence has shown that this conundrum is not restricted to RHAMM. In recent years a number of proteins that are classified as cytoplasmic (due to lack of Golgi/ER export sequence and membrane spanning sequence) are secreted by unconventional means and associate with integral receptors to influence cell signaling (Fig. 9.5) (Radisky et al., 2003; Nickel, 2005; Maxwell et al., 2008; Prudovsky et al., 2008). The signaling functions of

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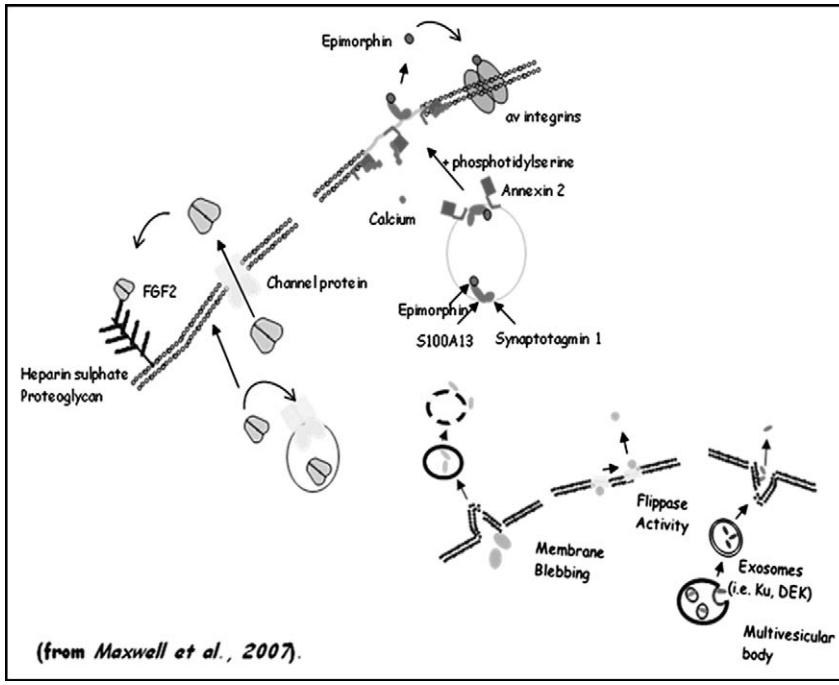

F0030 **FIGURE 9.5** Diagram of mechanisms for unconventional export of cytoplasmic proteins. Several mechanisms have been identified that permit release of cytoplasmic proteins in the absence of cell death. These include export through channels, via an export protein complex, or as a result of flippase activity, membrane blebbing or release of multi-vesicular bodies. Adapted from Maxwell et al. (2008).

these multifunctional or ''moonlighting'' proteins at the cell surface so far appear to be unrelated to their intracellular functions. RHAMM is one example of this class of proteins and others include epimorphin/syntaxin 2; galectins 1, 3, autocrine motility receptor, and bFGF1,2. Export mechanisms are still being characterized but to date appear to involve one of five types: passage through membrane channels, flipping through the plasma membrane via protein export complexes, membrane blebbing, flippase activity and exosomes (Fig. 9.5) (Maxwell et al., 2008).

p0125 Although the mechanisms for unconventional export of RHAMM have not yet been defined, it is secreted in response to specific stimuli as is the case with other unconventionally exported proteins. For example, TGFβ1 and hyaluronan both promote export of RHAMM to the extracellular compartment. The extent to which the extracellular functions of RHAMM are coordinated remain to be determined; however evidence suggests these apparently discrete pools of RHAMM are somehow linked. Further characterization of these RHAMM functions may provide unique paradigms

10009-STERN-9780123741783

for future structure/function analyses. At the very least, the puzzle of why the mitotic and motogenic functions are essential for wound repair (and appear to be utilized by tumors) but not for embryogenesis or adult homeostasis suggest that our current ability to relate function to structure is far from complete. The findings also imply that cell division and motility are differently regulated during embryogenesis/homeostasis compared to what occurs during wound repair/neoplasia. If so, our appreciation of the regulatory complexity of these fundamental cell functions is also still rudimentary. Although little insight into molecular mechanisms of these multiple documented RHAMM functions has been attained by proteomics, what has experimentation told us?

p0130 The motogenic signaling ability of RHAMM requires the cell surface display of this protein (Slevin et al., 2007; Turley et al., 2002; Tolg et al., 2006), which results from unconventional export in response to such motogenic factors as TGFB1 (Samuel et al., 1993) and hyaluronan (Gao et al., 2008). In fact, cell surface RHAMM alone is sufficient to promote migration over short time periods (e.g. 4 hours) since exogenous RHAMM added to $RHAMM^{-/-}$ fibroblasts was sufficient to rescue their ability to migrate in response to serum and hyaluronan Tolg et al., 2006). *In culture*, unconventional export of RHAMM also appears to be time (after plating) and cell density dependent (Turley, 1982; Hardwick et al., 1992). Stimulated export is a characteristic of unconventionally secreted proteins that differs from classical export through the Golgi-ER, which is constitutive in nature.

p0135 Exported RHAMM promotes activation of signaling cascades such as protein tyrosine kinases (e.g. c-abl, src, MST-1R,, FAK, Erk1,2, PI3 kinase and Ca^{++} fluxes in response to PDGF, hyaluronan, serum, bFGF and MSP (Turley et al., 2002; Greiner et al., 2006; Evanko et al., 2007; Naor et al., 2007; Slevin et al., 2007). RHAMM, which lacks membrane-spanning sequence and is a peripheral surface protein, appears to associate with integral receptors in order to promote activation of signaling cascades. As described above, RHAMM physically associates with integral receptors (e.g. CD44, and MST-1R) (Tolg et al., 2006; Hamilton et al., 2007; Manzanares et al., 2007). Although a co-association between cell surface RHAMM and CD44 has been described in aggressive human breast cancer cell lines and in dermal wound fibroblasts, it clearly partners with other as of yet unidentified cell surface receptors in the absence of CD44. The RHAMM/CD44 pairing promotes cell surface retention of CD44 and sustained activation of ERK1,2 possibly through formation of RHAMM/CD44 dimers. However, this partnering may not always transduce signaling in response to hyaluronan. For example, hyaluronan-promoted invasion of glioma cells into brain slices requires RHAMM but not CD44 even though individual tumor cells express both proteins (Akiyama et al., 2001). As another example, hyaluronan oligosaccharides promote protein

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tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin and ERK1,2 in endothelial cells (Lokeshwar and Selzer, 2000; Slevin et al., 2007; Gao et al., 2008). These signaling events require RHAMM although not necessarily involve CD44 but additional adhesion receptors such as integrins. Furthermore, although both CD44 and RHAMM are required for endothelial tube formation, CD44 is uniquely required for endothelial cell adhesion while only RHAMM is required for migration of endothelial cells in 2D culture (Savani et al., 2001). Importantly, RHAMM but not CD44 is required for bFGF-induced neo-vascularization in mice (Slevin et al., 2007).

p0140 These results indicate that CD44 and cell surface RHAMM share some functions, which likely require their physical association, but they also regulate distinct processes possibly in part by partnering with distinct membrane and/or signaling components. For example, the results and evidence discussed earlier that a cell surface form of RHAMM compensates for the genetic loss of CD44 in a hyaluronan dependent collagen-induced form of arthritis (Nedvetski et al., 2004) predict that RHAMM binds to additional hyaluronan receptors and/or to other integral membrane proteins. Indeed, the physical association of cell surface RHAMM with MST-1R in airway epithelia provides an example of a RHAMM partner that mediates growth factor (MSP1) regulated signaling (Manzanares et al., 2007).

p0145 To date, mitotic spindle/centrosome functions of intracellular RHAMM forms have been reported but the presence of RHAMM in multiple intracellular compartments (e.g. mitotic spindle, interphase microtubules, actin stress fibers, cell-substratum adhesions, nucleus, Fig. 9.6) and their association with disparate proteins such as BCL2 (Xu et al., 2003), calmodulin (Lynn et al., 2001), and glucose regulated proteins p78 and 75 (Kuwabara et al., 2006), predict additional intracellular functions. The mitotic spindle functions of RHAMM were first revealed when microinjected anti-RHAMM antibodies were observed to result in aberrant mitotic spindle formation (Maxwell et al., 2003). Consequently, several studies showed that RHAMM complexes with other mitotic spindle binding proteins including TPX2 and NuMa (Groen et al., 2004; Joukov et al., 2006; Pujana et al., 2007). Addition of recombinant RHAMM or use of anti-RHAMM antibodies to block endogenous RHAMM protein function results in aberrant mitotic spindle formation in extracts of Xenopus or HeLa cells (Joukov et al., 2006). Quantifying the amount of RHAMM in these cell extracts reveals that excess RHAMM protein results in formation of multiple and structurally aberrant spindles. The E3 ubiquitin ligase activity of BRCA1/BARD1 complexes attenuates the mitotic spindle functions of RHAMM by regulating the level of this protein at specific points of the cell cycle (Joukove et al., 2006). The proposed mechanisms by which cell surface RHAMM/CD44 activates motogenic signaling cascades and intracellular RHAMM/TPX2/BRCA1/

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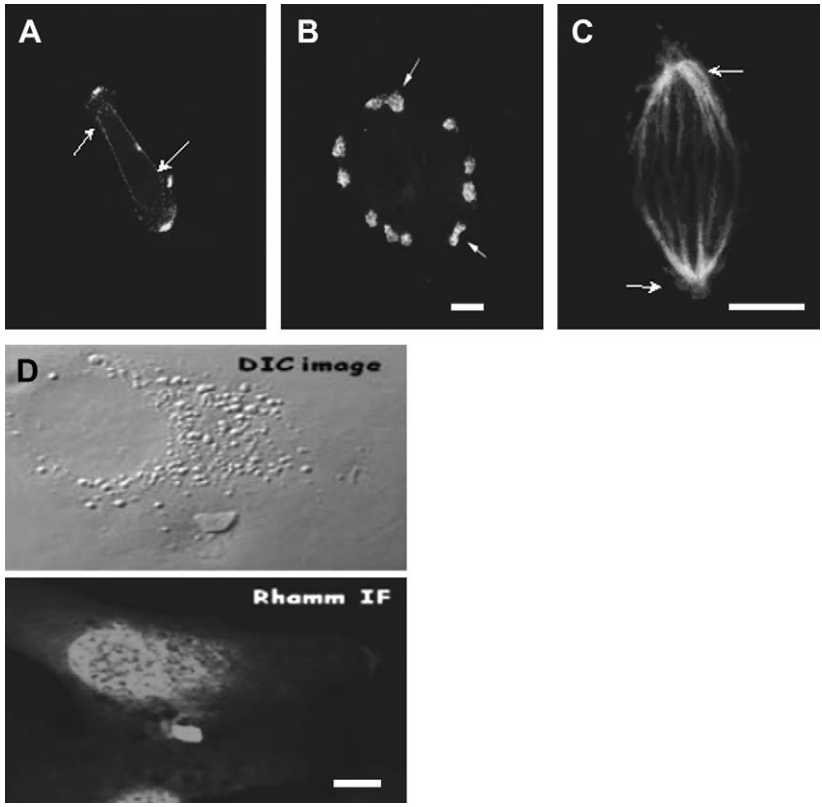

F0035 **FIGURE 9.6** RHAMM proteins are localized to multiple subcellular compartments. Confocal analyses of fibroblasts show that compartmentalization of RHAMM proteins change over time after subculture. Thus RHAMM protein is (**A**) initially found at the cell membrane as cells flatten onto the growth surface (red is RHAMM [arrow], green is cortactin) then (**B**) becomes distributed in podosomes (red is RHAMM, green is cortactin, arrows show colocalization of RHAMM and cortactin). Bar = 20μm (**C**) RHAMM decorates the apex of mitotic spindles (arrow, Bar = 5μm) of dividing cells (red is RHAMM and green is phospho-Erk1,2) and is often present in the (**D**) nuclei of interphase fibroblasts. Bar = 25μm.

BARD1 complexes regulate mitotic spindle integrity have recently been reviewed (Maxwell et al., 2008).

p0150 Subsequent studies using yeast two hybrid screens identified 28 additional protein partners for intracellular RHAMM of which several (BRCA1,2, CSPG6) were confirmed to associate with RHAMM in mammalian cells using immunoprecipitation assays (Pujana et al., 2007). From these and other published data a RHAMM "interactome" was constructed mainly of intracellular proteins that are components of various signaling pathways that impact upon cytoskeleton or centrosome integrity.

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These analyses have cemented a dogma that the cell surface signaling and mitotic spindle protein of RHAMM are distinct. But are the known intracellular and extracellular functions of RHAMM somehow linked? Certain studies (discussed above) suggest that some functions of extracellular and intracellular forms of RHAMM might be linked in mitosis. To reiterate, extracellular and intracellular RHAMM forms both affect progression through G2/M, and RHAMM levels increase during transit through the cell cycle. One way to develop testable models to address this question of relatedness is to use a systems biology approach to construct potential functional interactomes, which predict networks of interacting or functionally linked proteins. For example, this approach was used to construct a RHAMM/centrosome interactome implicated in spindle stability (Pujana et al., 2007). We constructed a similar interactome *in silico* using the Ingenuity Pathway Analysis Software-Complete Pathways Database (functional network program (http://www.ingenuity.com). With this program, we interrogated the functional interactions amongst reported RHAMM partner proteins, including both the intracellular and cell surface protein partners reviewed above. Four functional networks were identified but only one of these incorporated most of the well characterized RHAMM binding partners. This network (Fig. 9.7) includes RHAMM/HMMR, CD44, ERK1,2, BRCA1, 2, BARD1, RON, PDGFBB, PDGFR, MAD1L1, MAD2L1, HGS, XP06 and nuclear pore proteins. The functions of this network include cell cycle, cellular assembly and organization, consistent with the dynamic nature of both cell motility and mitotic spindle formation.

p0155 The two signaling pathways incorporated with the highest level of significance within this functional network account for the role of BRCA1 in DNA damage response ($p < 0.000180$) and PDGF signaling ($p < 0.001$). However, these interactomes do not include the previously published BRCA1 pathway in mitotic spindle formation/stability, although many of the components of this pathway are included (Fig. 9.7). The role for RHAMM in PDGF and other growth factor signaling is well documented (Turley et al., 2002; Slevin et al., 2007), as is an involvement of ERK1,2 in RHAMM controlled signaling (Turley et al., 2002; Slevin et al., 2007). The direct interaction between RHAMM and CD44 is represented in Fig. 9.7 and CD44 is predicted to link RHAMM to PDGFR. The top physiological system associated with this signaling network is connective tissue function ($p < 0.000005$) and the top disease category is cancer ($p < 0.000005$), in particular tumor morphology. Collectively, these results support numerous experimental data which suggest that both cell surface and intracellular RHAMM forms contribute to a functionally interconnected network that controls processes essential to cell motility and mitosis important for both connective tissue function and cancer progression.

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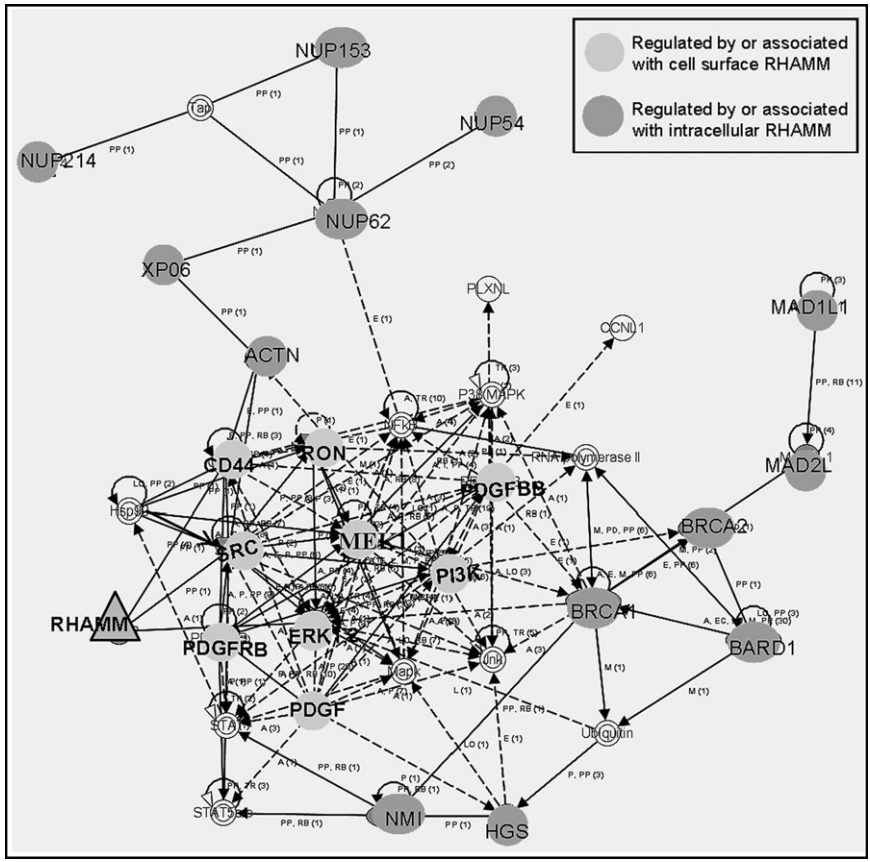

F0040 **FIGURE 9.7** Functional signaling network regulated by cell surface and intracellular RHAMM. Using published RHAMM protein intracellular and extracellular partners, functional networks constructed using Ingenuity Several networks were derived from this analysis, but the network shown here reveals that most of the proposed oncogenic effects of RHAMM can be functionally linked. For example, this network shows that CD44, RON, PDGF, MEK1/ERK1,2 are functionally linked to BRCA1,2, BARD 1 nuclear pore proteins and centrosomal proteins. The cell surface activities are shown in red and the intracellular activities are shown black. These results predict that at least some of the extracellular and intracellular functions of RHAMM may be coordinated. RHAMM (HMMR, receptor for hyaluronan mediated motility); RON (MSTR-1, macrophage stimulating 1 receptor); ACTN (alpha actinin); MEK1 (mitogen-activated protein kinase kinase); ERK1,2 (mitogen-activated protein kinase); PDGFRB (platelet derived growth factor receptor beta);PDGFBB (platelet derived growth factor beta dimer); SRC (Rous sarcoma oncogene); PI3K (phosphoinositide-3-kinase); CD44 [CD44 molecule (Indian blood group)]; BRCA1 (breast cancer 1); BRCA2 (breast cancer 2); BARD1 (BRCA1 associated RING domain 1); NMI (N-myc [and STAT] interactor); HGS (hepatocyte growth factor-regulated tyrosine kinase substrate); MAD2L (MAD2 mitotic arrest deficient-like 1); MAD1L1 (MAD1 mitotic arrest deficient-like 1); XPO6 (Exportin 6); NUP62 (nucleoporin 62); NUP54 (nucleoporin 54); NUP153 (nucleoporin 153) and NUP214 (nucleoporin 214).

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CONCLUSIONS

p0160 RHAMM is a gene that belongs to a multifunctional group of cytoplasmic proteins, which are unconventionally exported to the cell surface in response to specific stimuli. This process is highly active during response to injury and tumorigenesis and in certain situations/phenotypic backgrounds RHAMM, or structural variants of RHAMM, can act as an oncogene. RHAMM is poorly expressed in normal tissues but highly expressed and required for response-to-injury processes, in particular affecting inflammation and the subsequent mesenchymal response for repair. It is also found in numerous human tumors and hyperexpression of RHAMM is associated with poor clinical outcome of many these cancers. Cell surface RHAMM performs co-receptor functions by affecting signaling through hyaluronan and growth factor receptors while intracellular RHAMM is a mitotic spindle protein that interacts with BRCA1/BARD1 complexes to control mitotic spindle stability. Further analyses of the molecular mechanisms by which RHAMM regulates these processes will undoubtedly identify new ''inside–outside'' paradigms that control motility and mitosis and may lead to the identification of novel key signaling/structural nodes that can be targeted in the treatment of chronic inflammation or cancer.

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